Article

Combined application of rapamycin and atorvastatin improves lipid metabolism in apolipoprotein E-deficient mice with chronic kidney disease

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Running Title: RAPA plus ATV reduces cardiovascular risk in CKD

Keywords: Chronic kidney disease, Atherosclerosis, Rapamycin, Atorvastatin, Co-administration

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MATERIALS AND METHODS

Experimentally induced CKD mouse model (two-step procedure)

All animal studies were approved by the Institutional Animal Care and Use Committee of Ewha Womans University (IACUC-18-036). All experiments were carried out on female *ApoE*$^{-/-}$ mice at 8 weeks of age. The animals were maintained in a specific pathogen-free facility set on a 12-h light-dark cycle, and were given free access to chow diets and sterilized water. A two-step procedure was used to induce uremia. Briefly, the right kidney was dissected from the adrenal gland and surrounding fat through a 2-cm flank incision; it was then cauterized by Bovie High-Temperature Battery-Operated Cautery (Symmetry Surgical, TN, USA), except for 2 mm around the hilum. Two weeks later, the ureter and renal artery and vein were clipped using a surgical clip and left total nephrectomy was performed through a left flank incision. The sham operation used as a control comprised the decapsulation of both kidneys. After 2 weeks of recovery, the mice were divided into the CKD, RAPA, ATV, and RAPA+ATV groups and fed a Western diet (#D12097B; Research Diets, Inc., NJ, USA) for 10 weeks to induce atherogenesis. Where indicated, rapamycin (RAPA; 0.5 mg/kg) or/and atorvastatin (ATV; 10 mg/kg) were given by oral gavage for 5 days a week for 10 weeks. Rapamycin and atorvastatin were supplied by Pfizer.

Blood biochemistry

Whole blood was drawn through the retro-orbital plexus of each deeply anesthetized mouse using a heparin-treated capillary. Serum was separated by centrifugation at 1,500 g for 15 min and stored at -80 ℃ until analysis. Serum levels of BUN, creatinine, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using a Hitachi 7180 biochemistry autoanalyzer (Hitachi Ltd., Tokyo, Japan). The levels of calcium, phosphate, total protein, and albumin were also measured.

Atherosclerotic lesion analysis

Mice were euthanized with carbon dioxide inhalation, and the hearts and aortas were perfused through the left ventricle with ice-cold phosphate-buffered saline (PBS). Hearts, including the aortic roots, were embedded in frozen-section compound (3801480; Leica, IL, USA) and serially sectioned at 7 μm. For *en face* analysis, the aorta was opened along the longitudinal axis and pinned onto a black wax plate. For measurement of atherosclerotic plaque lesions, the aorta and the heart section were fixed with 10% formalin in PBS and stained using an Oil red O solution. The lesion areas were analyzed using the Axio Vision software (Carl Zeiss, Jena, Germany).

RNA isolation and quantitative real-time PCR

Total RNA from tissue samples was prepared with the TRIzol reagent (Gibco, CA, USA), and cDNA was synthesized with the Maxime^{TM} RT-PCR PreMix (iNtRON Biotechnology, Korea). Quantitative real-time PCR (SYBR® FAST, Kappa Biosystems, MA, USA) was conducted to determine the relative levels of mRNA using a 7700 sequence detector (Applied Biosystems, CA, USA) and primers for the target mouse genes (Supplemenatry Table 1). The data were normalized to the mRNA level of *Gapdh* in each reaction.

Western Blot Analysis

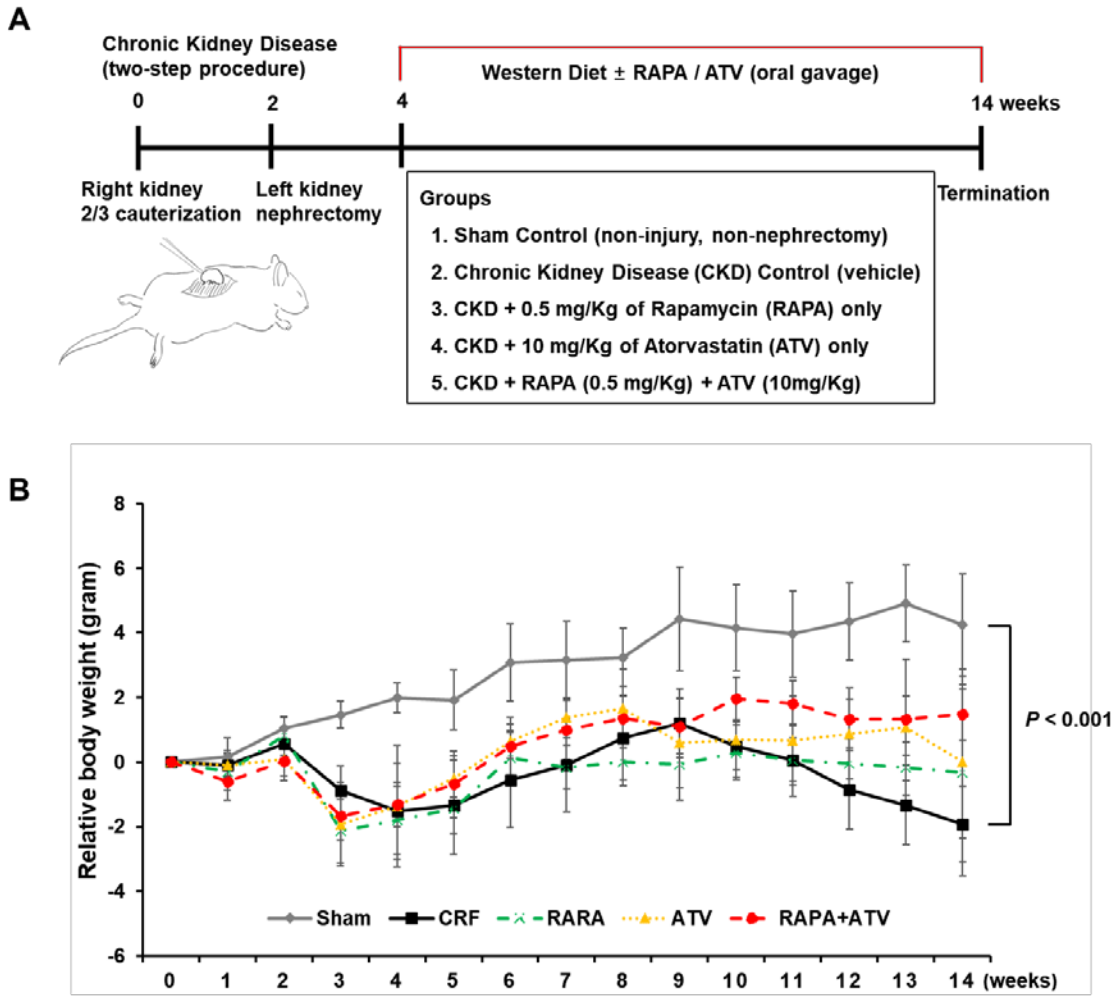
Total protein was extracted from tissue samples using EzRIPA Lysis buffer containing protease and phosphatase inhibitor cocktail (ATTO, Tokyo, Japan). For analysis of target protein expression, proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was incubated in primary antibodies at 4 ℃ for overnight, and with secondary antibodies for 2 hours at room temperature. Immunoreactive band were visualized and quantified. Antibodies for target protein were purchased as followed: anti-LXRα (Abcam, Cambridge, UK); anti-Cyp7a1, anti-PPARγ, anti-IL6 and anti-VCAM1 (Santa Cruz Biotechnology, CA, USA); anti-ABCG1 (Novus Biologicals, CO, USA); anti-ApoA1 (Biodesign, ME,

USA); anti-GAPDH and HRP-conjugated goat anti-mouse/rabbit IgG antibodies (GeneTex, TX, USA).

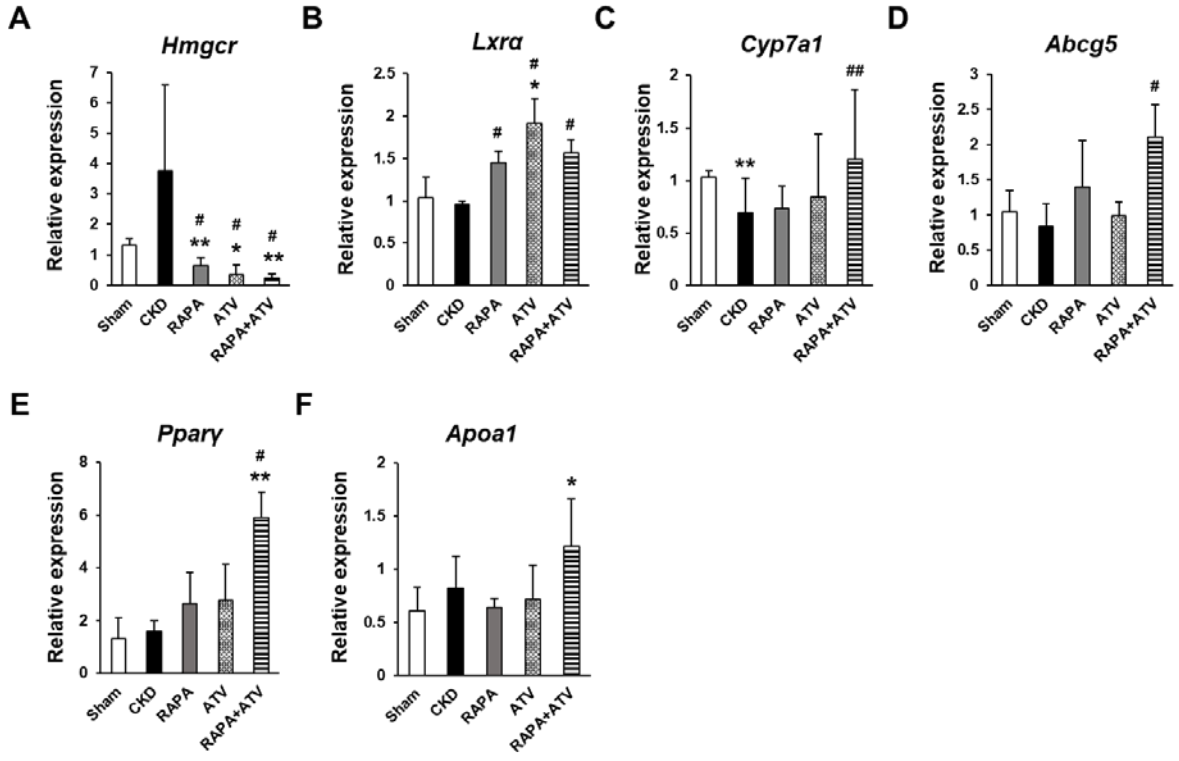
Statistical analysis

Continuous variables are expressed as the mean ± standard deviation and were compared using the Mann-Whiney *U* test. Categorical variables were tested with Fisher's exact tests and are expressed as counts and percentages. Body weights were analyzed using a repeated-measures ANOVA followed by Bonferroni's post-hoc test. Analyses were performed with the SPSS Statistical Analytics software (IBM Analytics, NY, USA).

SUPPLEMENTARY FIGURES



Supplementary Figure 1. (A) Schematic presentation of the experimental protocol. (B) Changes in the body weights of experimental animals.



Supplementary Figure 2. mRNA expression levels of (A) cholesterol metabolism-related genes, (B, C) cholesterol transport-related genes, (D) bile acid biosynthesis genes, and (E, F) lipid and glucose metabolism-related genes in the livers of the indicated groups (n=5-7 per group). Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the CKD group.

SUPPLEMENTARY TABLE

Supplementary Table 1. Primer sequences

	Sequence (5' -> 3')	
Gene	**Forward primer**	**Reverse primer**
Hmgcr	TTTCTAGAGCGAGTGCATTAGCA	GATTGCCATTCCACGAGCTATAT-3’
Lxrα	GATGTTTCTCCTGATTCTGCAAC	AGGACTTGAGGAGGTGAGGAC
Abcg5	CCTGCTGAGGCGAGTAACAA	TGGCACCCACAAGCTGATAG
Cyp7a1	CACTCTACACCTTGAGGATGG	GACATATTGTAGCTCCTGATCC
Pparγ	AGATTCAGAAGAAGAACCGGAAC	CCGATCTCCACAGCAAATTATAG
Apoa1	GCATGCGCACACACGTAGACTCTCT	CGTCTCCAGCATGGGCATCAGACTA
Tnf-α	TGGCCCAGACCCTCACACTCAG	ACCCATCGGCTGGCACCACT
Il-6	CTTCCATCCAGTTGCCTTCTTG	AATTAAGCCTCCGACTTGTGAAG
Il-1β	GGAGAACCAAGCAACACAAAATA	TGGGGAACTCTGCAGACTCAAAC
Il-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Il-4	GAATGTACCAGGAGCCATATC	CTCAGTACTACGATGAATCCA
Gapdh	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG